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Brevetoxin Depresses Synaptic Transmission in Guinea Pig Hippocampal Slices

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Hippocampal slice Sodium channel Field potential Brevetoxin Electrophysiology Synaptic transmission

BREVETOXINS, neurotoxins isolated from the unarmored marine dinoflagellate *Ptychodiscus brevis*, are responsible for red tides along Florida's Gulf Coast (6). They cause massive fish kills and are a public health concern due to occasional human illness. Inhalation of contaminated seaspray can cause respiratory distress, and neurotoxic shellfish poisoning can result from consumption of shellfish which have ingested *P. brevis* (7). Brevetoxins are complex polycyclic ethers with high lipid solubility; at least eight active toxins, designated PbTx-1 through PbTx-8, have been isolated from *P. brevis* cultures (20). The toxins incorporate one of two hydrocarbon backbones designated types 1 and 2, whose structures have been determined (16,27).

Brevetoxins exert a variety of actions in vivo. Fish poisoned with the toxins exhibit violent, uncoordinated movements, convulsions, paralysis, and respiratory failure. In mice, symptoms consist of irritability, hindquarter paralysis, and respiratory impairment (6). Physiological effects include both central and peripheral components. Respiration and cardiovascular function are severely depressed (6,9,10,13,21). Spontaneous muscular contractions are manifested as fasciculations, twitching, leaping, and, at high doses, somatomotor seizures (7,9,10,21). A recently developed antibrevetoxin antibody relieved peripheral symptoms of brevetoxin intoxication within minutes, but neurological signs persisted for several hours (31). These results suggest that the antibody does not penetrate the blood–brain barrier; the pronounced central effects of the toxin are thereby revealed.

In vitro, brevetoxins exert several actions on isolated peripheral neuromuscular preparations that provide clues to their cellular mechanism of action. At frog and rat neuromuscular junctions the toxins depolarize muscle membranes and increase miniature endplate potential (MEPP) frequency (5,11,14,28,35). Both effects are blocked by tetrodotoxin (5,28,35), and low-Ca^{2+} solutions block the increased MEPP frequency (5). A variety of related brevetoxin actions have been observed in the rat phrenic nerve-hemidiaphragm preparation. First, muscle twitch elicited by stimulation of the phrenic nerve was blocked by brevetoxin at concentrations lower than those required to block the twitch elicited by direct muscle stimulation (8,11,14). Second, the evoked endplate potential (EPP) failed abruptly in the presence of toxin, without first displaying any graded decrement (11,14). Third, disappearance of the compound action potential in the phrenic nerve paralleled the loss of the EPP and the indirect twitch (11). At higher concentrations, the toxin blocked directly elicited muscle action potentials (11), decreased muscle membrane input resistance (25), and depolarized the resting membrane (11,14,28). In a more recent study (33) of mouse motor

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nerve terminals, nanomolar concentrations of PbTx-2 increased sodium and potassium currents. Micromolar concentrations of the toxin decreased sodium, potassium, and calcium currents.

The present study was undertaken to investigate the effects of brevetoxins in the central nervous system (CNS) in vitro. The hippocampal slice was chosen for this study because somatomotor seizures have been reported during brevetoxin intoxication (10) and the hippocampus has been implicated in initiation and propagation of seizures (30). We have applied PbTx-3, which has the more stable type 1 structure (6), to hippocampal slices and demonstrated that the toxin depresses electrically evoked responses in CA1 pyramidal cells. These results have been presented previously in abstract form (3).

METHOD

Slice Preparation and Superfusion

Male guinea pigs (*Cavia porcellus*), 250–300 g, were maintained under an AAALAC accredited animal care and use program. Standard techniques for slice preparation and recording have been described previously (4,17). Briefly, guinea pigs were deeply anesthetized with isoflurane and decapitated; the brains were removed and the hippocampi were dissected free. Transverse slices of hippocampus, 400 μm thick, were cut with a tissue chopper. Slices were placed in an incubation chamber (17) and maintained at room temperature in oxygenated artificial cerebrospinal fluid (ACSF) for at least 1 h before recordings were performed. The recording chamber was based on a design described previously (17). Experiments were performed on 55 slices from 40 animals; one to three slices were used per animal.

The standard ACSF contained (in mM) NaCl 124, KCl 5, $MgSO_4$ 2, $CaCl_2$ 2, NaH_2PO_4 1.25, $NaHCO_3$ 26, glucose 10, equilibrated with 95% O_2–5% CO_2, pH 7.4. High-K^+ and high-Ca^{2+} solutions were made by uncompensated addition of KCl and $CaCl_2$, respectively, to yield the indicated concentrations in modified ACSFs. Tetraethylammonium (TEA^+) chloride, 4-aminopyridine (4-AP), and N-methyl-D-aspartate (NMDA) were purchased from Sigma Chemical Company and isoflurane from Anaquest. Purified PbTx-3 was obtained from the U.S. Army Medical Research Institute for Infectious Diseases (Ft. Detrick, MD). The toxin was dissolved in a 1:1:8 mixture of chloroform: methanol:ethanol and stored at −14°C. Aliquots of the stock solution were added directly to ACSF to give working toxin solutions. The total concentration of organic solvents was less than 0.03% (v/v). Equivalent concentrations of vehicle alone had no measurable effect on the slices. Agents were bath-applied, alone or in combinations. Slices were totally immersed and continuously superfused with oxygenated ACSF at 32°C.

Recording and Stimulation

A low-resistance (1–4 MΩ) glass microelectrode filled with ACSF was placed in the CA1 pyramidal cell layer for extracellular recording of neuronal activity in response to both orthodromic and antidromic stimulation. Orthodromic electrical stimulation was presented to Schaffer collaterals and commissural fibers with a monopolar tungsten electrode placed in stratum radiatum. Antidromic responses were evoked by stimulation of the alveus with a concentric bipolar electrode. Supramaximal paired pulses, 70 ms apart and 10 μs in duration, were presented every 4 s, eliciting the first (FP1) and second (FP2) field potentials. The paired-pulse paradigm revealed frequency potentiation of FP2 in response to orthodromic stimulation in control slices; FP2 was also more sensitive to drug treatments. Field potentials were digitized and stored on a personal computer using pClamp software (Axon Instruments, Inc.). EC_{50} values were calculated using GraphPAD InPlot (GraphPAD Software) by nonlinear regression analysis of the percent block of FP2 population spike amplitude versus log concentration of the toxin.

RESULTS

Effects of Brevetoxin on Orthodromically Evoked Responses

Orthodromically evoked field potentials from a typical experiment under control conditions are shown in Fig. 1A. Superfusion of this slice with ACSF containing PbTx-3 (100 nM) produced characteristic changes in electrically evoked responses. Exposure to toxin caused hyperexcitability to develop in about 50% of the slices. Hyperexcitability was revealed by additional population spikes, which were more often present in FP2 (Fig. 1B). With continued PbTx-3 exposure, orthodromically evoked responses were progressively depressed, eventually becoming blocked at toxin concentrations ≥100 nM (Fig. 1C and D). Onset of the toxin effect was slow, requiring 14–60 min to develop fully. Washout was even slower, requiring nearly 3 h for full recovery of the evoked response (Fig. 1F). Effects of PbTx-3 were concentration dependent, in that hyperexcitability was more likely to develop in lower (10–100 nM) toxin concentrations.

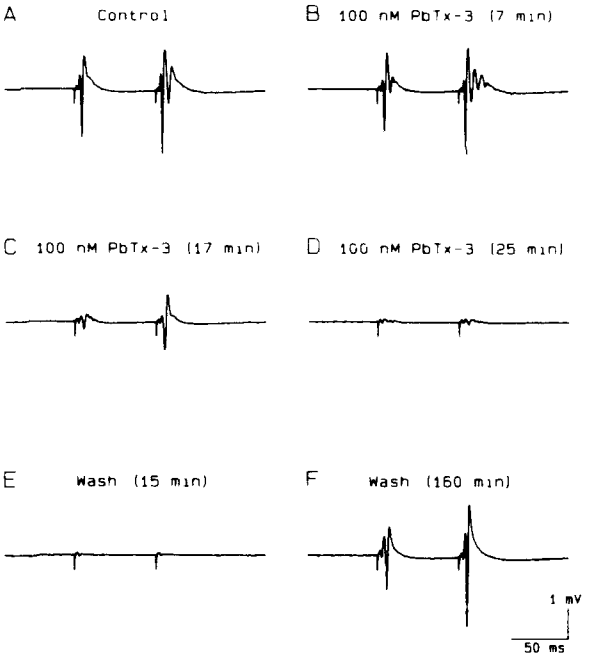

FIG. 1. Brevetoxin (PbTx-3) depressed orthodromic extracellular field potentials (FPs) recorded from the pyramidal cell body layer of area CA1 of a hippocampal slice in a typical experiment. Similar results were obtained in 23 additional slices. Paired pulses were presented, 70 ms apart, every 4 s. (A) Normal artificial cerebrospinal fluid (ACSF) control. (B) A 7-min exposure to 100 nM PbTx-3 caused increased excitability. The hyperexcitability was revealed by additional population spikes, particularly in FP2. This phenomenon occurred with greater regularity at lower concentrations of toxin. (C) A 17-min exposure to toxin caused depression of the evoked responses. The FP2 population spike was depressed by 50%. (D) After a 25-min exposure, the responses were almost completely blocked. (E) No recovery was seen after washing with normal ACSF for 15 min. (F) Responses recovered completely after a 160-min wash.

Depression of responses also developed more rapidly, and washout was slower, at higher PbTx-3 concentrations (Fig. 5).

The concentration-response relationship for depression of the orthodromically evoked FP2 population spike by PbTx-3 is shown in Fig. 2. The peak amplitude of the FP2 population spike was chosen for quantitative analysis because its amplitude was larger than that of the FP1 spike and it was last to be completely blocked. The time to maximum depression of the FP2 population spike was concentration dependent, being longest at the lowest concentrations. The IC_{50} for steady-state depression was 37.5 nM with a Hill coefficient of 2.8.

Contrast Between Effects on Orthodromic and Antidromic Responses

Effects of PbTx-3 on responses to orthodromic and antidromic stimulation were distinctly different. Orthodromic responses were always depressed before antidromic responses at equal PbTx-3 concentrations (Figs. 3 and 4). The orthodromic response became fully depressed with continued exposure to 100 nM PbTx-3 (Fig. 3C and E), although the antidromic response was unaffected (Fig. 3D and F). The toxin exposure was continued up to 1 h in this slice, yet the antidromic response remained at the control amplitude. The orthodromic response was again the first to be depressed by exposure to higher concentrations (>100 nM) of PbTx-3 (Fig. 4). After a short exposure to 300 nM PbTx-3 when the orthodromic response was fully suppressed (Fig 4C), the antidromic response was still 40% of control (Fig. 4D). Eventually both responses were completely blocked (Fig. 4E and F).

The time course of depression of electrically evoked responses is shown in Fig. 5. When a slice was exposed to 300 nM PbTx-3, depression of both orthodromic and antidromic responses began almost immediately. However, the orthodromic response was completely suppressed within 12 min, whereas complete block of the antidromic response required PbTx-3 exposure for 45 min. Exposure of a different slice to 100 nM toxin led to complete blockade of the orthodromic response within 35 min.

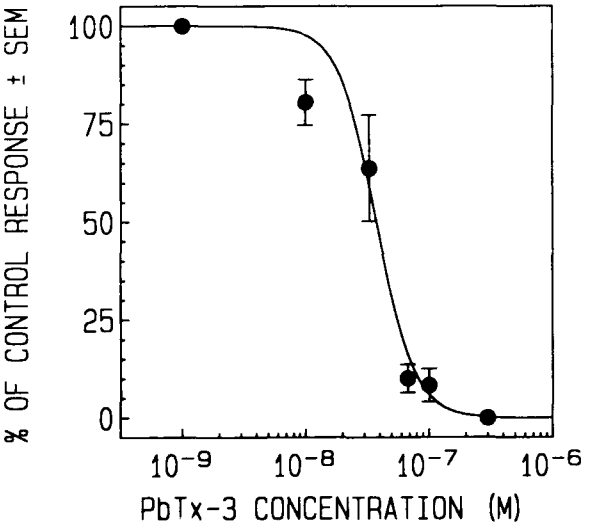

FIG. 2. Steady-state depression of the orthodromically evoked FP2 population spike by PbTx-3. The IC_{50} was 37.5 nM and the Hill coefficient was 2.8. Vertical bars represent SEM (n = 3–24 slices).

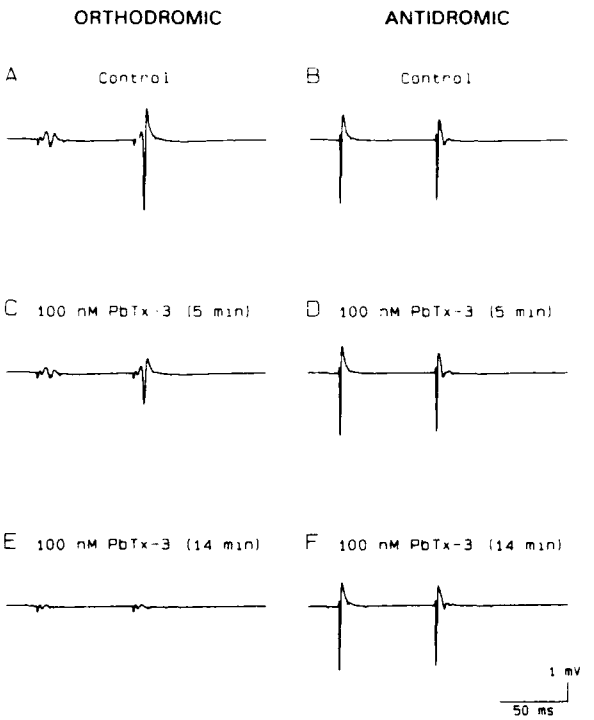

FIG. 3. Low concentrations of PbTx-3 caused depression of orthodromically evoked responses, although antidromic responses were not depressed in a typical experiment. Similar results were obtained with 20 additional slices. Orthodromic responses are shown on the left and antidromic on the right for each time point after toxin application. (A) Control orthodromic response in normal ACSF. (B) Antidromic control. (C) A 5-min exposure to 100 nM PbTx-3 caused more than 50% depression of the orthodromic response. (D) The antidromic response was unaffected at 5 min. (E) After a 14-min exposure to toxin, the orthodromic response was completely blocked. (F) At 14 min the antidromic response was still not depressed.

The antidromic response, however, was never depressed more than 20%, despite continued toxin exposure for 10 min after the orthodromic response had disappeared. Depression of the orthodromic response by both 100 nM and 300 nM brevetoxin followed a similar time course; the major difference was the more rapid onset with 300 nM toxin. Antidromic responses in the presence of both 100 nM and 300 nM toxin typically declined more slowly than the corresponding orthodromic responses. The antidromic response in the presence of 100 nM PbTx-3 remained stable after 25 min, whereas this response was progressively depressed to zero in 300 nM toxin.

Comparison of Effects of PbTx-3 with Other Treatments

To assess how PbTx-3 exerts its effects, the actions of other depolarizing agents on orthodromic and antidromic responses were compared with those of PbTx-3. Bath application of NMDA, like PbTx-3, caused depression of the orthodromic response before any significant change in the antidromic response was observed (Fig. 6E and F). Exposure to NMDA for an additional 2 min in this experiment also depressed the antidromic response by 90% (not shown). However, NMDA, unlike PbTx-3, invariably caused hyperexcitability in the orthodromic re-

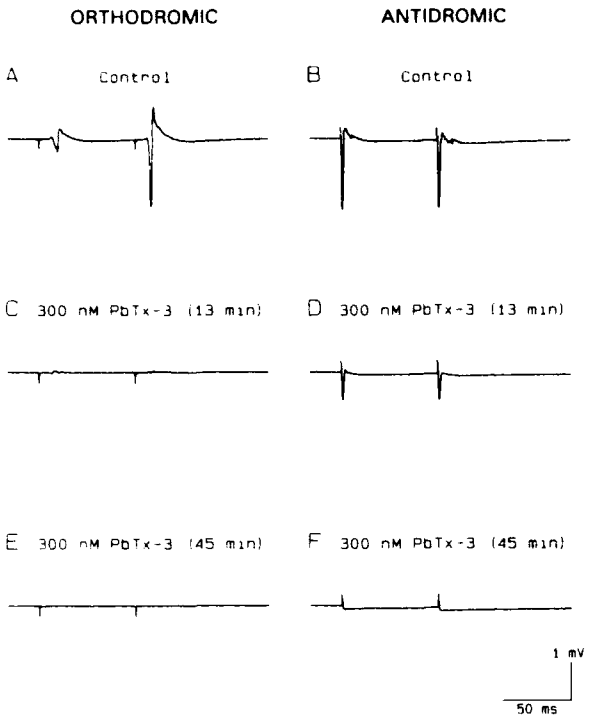

FIG. 4. With higher concentrations of toxin and longer exposure, antidromic responses were also depressed in a slice typical of six tested. Orthodromic responses are on the left and antidromic on the right at each time point. (A) Control orthodromic response. (B) Antidromic control response. (C) The orthodromic response was completely blocked after a 13-min exposure to 300 nM PbTx-3. (D) At 13 min the antidromic response was partially depressed. (E) As expected, the orthodromic response was still blocked after a 45-min exposure to toxin. (F) At 45 min the antidromic response was also blocked.

sponse prior to inhibition. In Fig. 6C, 12 μM NMDA caused numerous afterdischarges in both FP1 and FP2, and some remaining hyperexcitability could still be seen in Fig. 6E after the population spikes had been completely blocked. These results were similar to those obtained in a previous study (4). This degree of excitability was not observed with any concentration of PbTx-3 in our experiments.

Effects of high K^+ ACSF were similar, in that this treatment also consistently caused hyperexcitability in the orthodromically evoked response (Fig. 7C). Both orthodromic and antidromic responses were depressed after a 4-min exposure to 15 mM K^+ ACSF as shown in Fig. 7C and D. As with PbTx-3 and NMDA, the orthodromic response was always depressed before the antidromic. In Fig. 7C the orthodromic FP2 population spike was fully suppressed, whereas the antidromic FP2 population spike was depressed by only 40% (Fig. 7D). After an additional 1-min exposure to 15 mM K^+ both orthodromic and antidromic responses were completely blocked. The orthodromic response was also more completely inhibited in 10 mM K^+ than was the antidromic; however, neither response was completely blocked at this K^+ concentration. The onset of both NMDA and high K^+ effects occurred with much shorter latencies than did those of PbTx-3.

Attempts to Antagonize the Effects of PbTx-3

High Ca^{2+} ACSF was used in an attempt to enhance transmitter release and thereby counteract the depressant effect of PbTx-3 on the orthodromic response. Four mM Ca^{2+} was not effective in preventing the toxin's depression of evoked responses. The limit of solubility of Ca^{2+} in our bicarbonate-buffered ACSF was 4 mM. In an attempt to further elevate Ca^{2+} concentration, Tris or HEPES were substituted for bicarbonate while bubbling the solution with pure O_2. Evoked responses were depressed by both Tris- and HEPES-buffered solutions (data not shown).

Both 4-AP and TEA^+ were used in an attempt to block presynaptic K^+ currents and thereby enhance transmitter release. 4-AP (50 μM), 5 mM TEA^+, and 5 mM TEA^+ in combination with 4 mM Ca^{2+} were ineffective in preventing depression of evoked responses by PbTx-3 (data not shown). Both 4-AP and TEA^+ caused the expected increase in excitability of hippocampal CA1 neurons.

DISCUSSION

There are several reasons for expecting brevetoxins to act on the CNS. The specific site of brevetoxin action has been proposed to be the α subunit of the voltage-sensitive sodium channel (VSSC) of excitable cells (32), and all actions of the toxins have been ascribed to Na^+ entry through activated channels (29,36). Furthermore, brevetoxins have high lipid solubility, enabling them to penetrate the blood–brain barrier, and a number of centrally mediated symptoms have been described (6).

Results of our experiments in hippocampal neurons correlate well with findings of other investigators on the peripheral actions of PbTx-3. Compound action potentials of phrenic nerves were reduced by 30% in the presence of 50 nM PbTx-3 and completely blocked by 100 nM toxin; the reduction of the action potential amplitude paralleled the loss of the nerve-evoked twitch and of the EPP (11,26). These results are in agreement with our demonstration that PbTx-3 concentrations up to 100 nM caused selective depression of orthodromically evoked responses. In skeletal muscle, toxin concentrations $\geq$500 nM were required to block twitches elicited by direct muscle stimulation (11). In the hippocampus, higher concentrations of toxin (up to 300 nM)

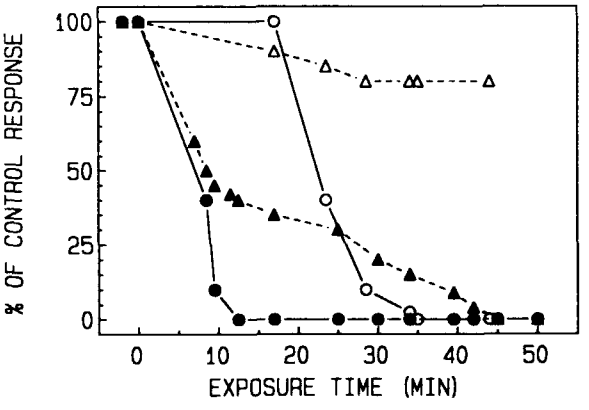

FIG. 5. Time course of depression of responses to orthodromic and antidromic stimulation by two concentrations of PbTx-3. Exposure of a typical slice to 300 nM PbTx-3 caused depression of both orthodromic (●) and antidromic (▲) responses. Exposure of a different slice to 100 nM toxin eventually caused complete depression of the orthodromic (○), but not the antidromic (△), response.

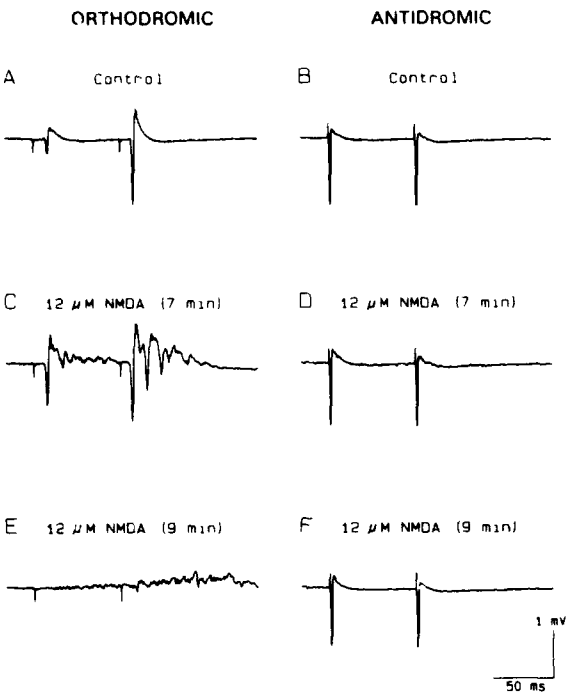

FIG. 6. NMDA, like PbTx-3, caused depression of orthodromic responses before antidromic responses in two slices tested: data from one are shown here. Orthodromic responses are on the left and antidromic on the right at each time point. (A) Control orthodromic response. (B) Antidromic control response. (C) Exposure to 12 μM NMDA invariably caused hyperexcitability, with numerous epileptiform afterdischarges, before the orthodromic responses were depressed. (D) After a 7-min exposure to NMDA, antidromic responses revealed neither hyperexcitability nor depression. (E) A 9-min exposure to NMDA caused almost complete depression of the orthodromically evoked response, with some instability evident. (F) At 9 min the antidromic response was still not depressed. With further exposure to NMDA, this response was also depressed.

were needed to depress responses evoked by antidromic than by orthodromic stimulation. We did not use toxin concentrations as high as those reported to be necessary to decrease fast calcium currents in mouse motor nerve terminals (>550 nM) (33). The long wash times required for recovery of responses in hippocampal slices are consistent with the high lipid solubility of the toxin and are also characteristic of peripheral actions of PbTx-3 (21).

Effects of PbTx-3 on CA1 pyramidal neurons in hippocampal slices are similar in some ways to those of NMDA and high K^+ ACSF. NMDA is an excitatory amino acid, which at low concentrations and short exposure times causes small depolarizations of hippocampal neurons and prominent afterdischarges in orthodromically evoked responses. Higher NMDA concentrations and longer exposure times result in more extensive depolarization of neurons and block of evoked responses, as shown here and previously (18,19). High K^+ ACSF depolarizes large pyramidal neurons as predicted by the Goldman equation (24), with accompanying hyperexcitability. We report here that PbTx-3, NMDA and high-K^+ ACSF all depressed orthodromic responses earlier and more completely than antidromic responses. It, therefore, seems probable that PbTx-3, like NMDA and high K^+ ACSF, acts by depolarizing hippocampal neurons. This interpretation is supported by the fact that the principal in vitro effect of brevetoxins on peripheral nerves is depolarization of their membranes in a concentration-dependent manner (6,29,36). It is possible that the toxin could enter the presynaptic nerve terminal, causing spontaneous release and depletion of transmitter by some mechanism other than membrane depolarization. However, in at least 50% of slices, particularly at higher toxin concentrations, there was no sign of the increased hyperexcitability that might be expected to result from spontaneous transmitter release. Based on the evidence we have, the most parsimonious interpretation is depolarization of the presynaptic elements.

The finding that PbTx-3 depresses orthodromic responses earlier than antidromic responses is intriguing. In fact, at concentrations ≤100 nM, PbTx-3 blocked orthodromic responses with little effect on antidromically evoked potentials. The explanation may be analogous to one proposed to account for the loss of indirect twitch before direct twitch in skeletal muscle (6,14). Brevetoxin's activation of VSSCs would depolarize nerve terminals, decreasing the amplitude of the nerve terminal action potential. Invasion of the action potential into the nerve terminal

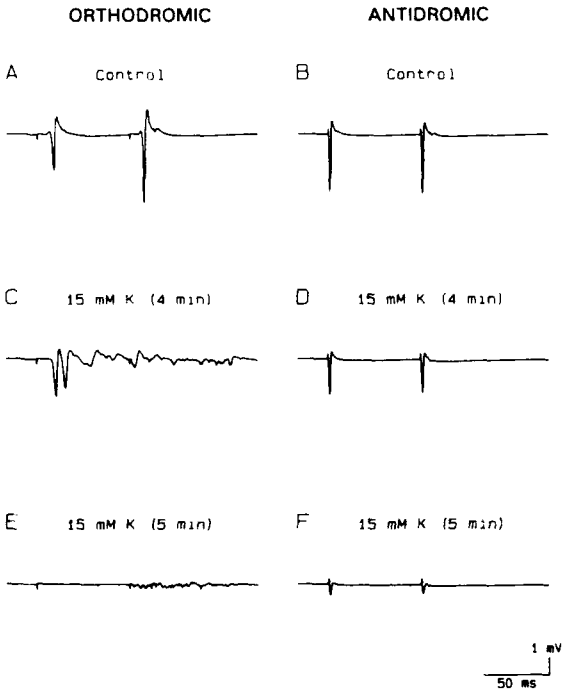

FIG. 7. High K^+ ACSF caused hyperexcitability and then depression of orthodromic responses in an experiment typical of four slices tested. Antidromic responses were also depressed, usually after the orthodromic. Orthodromic responses are displayed on the left and antidromic on the right at each time point. (A) Orthodromic response in normal ACSF. (B) Control antidromic response. (C) After a 4-min exposure to 15 mM K^+, the orthodromic response displayed hyperexcitability, with epileptiform afterdischarges present. The FP2 population spike was suppressed by the afterdischarges following FP1. (D) At this time the antidromic response was depressed by 40%. (E) One minute later, only small unitary activity remained of the orthodromic response. (F) The antidromic response was reduced more than in D above. With further exposure to high K^+, this response was also completely blocked.

would be impeded by depolarization of the presynaptic membrane, and neurotransmitter release would then be inhibited. Some investigators (12,15) have proposed that neuronal depolarization may cause presynaptic fiber branch point block, due to the lower safety factor at branch points. A modest depolarization-induced block of VSSCs might, therefore, inhibit propagation of impulses to nerve terminals and depress transmitter release, leaving conduction intact in the axon near the cell body. Further toxin action on additional VSSCs could subsequently block antidromic transmission in addition to orthodromic.

The paucity of epileptiform activity observed in hippocampal slices was somewhat unexpected in light of the seizures and neurological signs seen in vivo. The frequent repetitive stimulation, used in the present study would be expected to prevent spontaneous ictal-like discharges (2), perhaps due to strong synaptic inhibition present in the hippocampus (1), so the lack of spontaneous discharges was not surprising. However, PbTx-3 did not cause the incidence or degree of epileptiform activity seen in the presence of either NMDA or high K^+ ACSF. NMDA is a purely excitatory amino acid, so its application would be expected to cause only excitation of CA1 pyramidal cells. Like high K^+ solutions, PbTx-3 increases spontaneous MEPP frequency in muscle (5,11,14,28,35) and releases both excitatory and inhibitory neurotransmitters from rat cerebral cortical synaptosomes (23). Both agents might be expected to have similar effects in hippocampal slices, yet only high K^+ solutions consistently caused marked epileptiform activity.

None of the attempted treatments for reversal of brevetoxin's actions were effective. Some explanations for these findings can be suggested. Elevation of Ca^{2+} to 4.8 mM has been shown to reduce population spike amplitude (22). The reason may be that binding of glutamate, the major excitatory amino acid in the hippocampus, to its postsynaptic receptor is depressed at Ca^{2+} concentrations above 2.5 mM (22). TEA^+ has recently been demonstrated to decrease NMDA receptor channel conductance and frequency of channel openings in cultured mammalian neurons (34). In this respect, 4-AP may be similar to TEA^+. These side effects would thus prevent the treatments from reversing the actions of PbTx-3. Furthermore, if failure of orthodromic transmission in brevetoxin is all or none at individual presynaptic terminals, then measures intended to boost transmission initiated by weak presynaptic spikes would have little effect on the integrated field potentials. Therefore, other approaches may have to be explored for potential treatment of brevetoxin poisoning.

This is the first report of the direct application of brevetoxin to central nervous tissue and its effects on cellular function. The actions and potency of PbTx-3 were similar to those reported for the phrenic nerve-diaphragm preparation (11). In vivo, CNS effects persisted after antibrevetoxin antibody relieved peripheral symptoms of intoxication (31). This indicates that the concentrations of brevetoxin used in our experiments in vitro were similar to those used in the work cited above. The increase in excitability induced by PbTx-3 in the hippocampal slice is not as great as expected from the CNS symptoms and seizures reported from brevetoxin intoxication, and from the fact that the toxins cause activation of VSSCs. However, PbTx-3 did cause profound depression of evoked responses in CA1 hippocampal pyramidal cells at nanomolar concentrations, suggesting that this action may be a major component of systemic brevetoxin poisoning.

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Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the "Guide for the Care and Use of Laboratory Animals," NIH Publication 85-23, 1985.

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